

CRISPR technology: The engine that drives cancer therapy

Mingtao Zhang^{a,b,1}, Emily A. Eshraghian^{c,1}, Omar Al Jammal^c, Zhibi Zhang^{d,*},
Xiao Zhu^{a,b,e,f,**}

^a Guangdong Key Laboratory for Research and Development of Natural Drugs, The Marine Biomedical Research Institute, Guangdong Medical University, Zhanjiang, China

^b Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang), Zhanjiang, China

^c Department of Family Medicine and Public Health, School of Medicine, University of California San Diego, CA 92093, USA ^d Biomedical Engineering Research Center, Kunming Medical University, Kunming, China ^e The Marine Biomedical Research Institute of Guangdong Zhanjiang, Zhanjiang, China ^f The Key Lab of Zhanjiang for R&D Marine Microbial Resources in the Beibu Gulf Rim, Guangdong Medical University, Zhanjiang, China

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ABSTRACT

CRISPR gene editing technology belongs to the third generation of gene editing technology. Since its discovery, it has attracted the attention of a large number of researchers. Investigators have published a series of academic articles and obtained breakthrough research results through in-depth research. In recent years, this technology has developed rapidly and been widely applied in many fields, especially in medicine. This review focuses on concepts of CRISPR gene editing technology, its application in cancer treatments, its existing limitations, and the new progress in recent years for detailed analysis and sharing.

1. Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a natural defense system unique to bacteria and archaea that aids in resisting invasion of viruses or exogenous plasmids [1–3]. Several types of CRISPR-associated (Cas) proteins have been identified, each with various functionality; the CRISPR/Cas system can be divided into six types. Type II systems, with the aid of Cas9 protein and single guide RNA (sgRNA), are known for their ability to edit target molecules. The type II CRISPR/Cas9 system is currently the main protein utilized for genome editing [4–6].

There are two main classes of Cas protein, as characterized by their functional and structural distinctions. Class 1 systems use a complex of multi-Cas proteins while class 2 systems use a single large Cas protein; both classes are used for degradation of foreign nucleic acids. Of these, there are two main Cas proteins which have received wide attention: Cas1-Cas2 complex and Cas9. The Cas1-Cas2 complex functions to collect genetic material of the invading virus or phage to produce memory. Cas9 protein is an effector protein which, under the guidance

of specific guide RNA (gRNA) or CRISPR RNA (crRNA) recognizes and cuts matching foreign DNA or RNA, so that re-invading pathogens cannot replicate [7,8].

1.1. Principle of the type II CRISPR Cas9 system, molecular mechanisms and

The type II CRISPR Cas9 system is made of Cas9 protein, crRNA, and trans-activating RNA. The Cas9 protein functions to snip double-stranded DNA at the sequence target via crRNA matching. By artificial design, crRNA and tracrRNA can be transformed into sgRNA with guiding function, which can guide Cas9 to perform fixed-point cutting of DNA, gene knockout, and insertion (Fig. 1) [9,10].

At the molecular level, bacteria integrate a sequence of foreign genes from the first invading viruses into their CRISPR spacer. When the virus re-invades, CRISPR performs gene transcription in order to generate precursor crRNA, which is further processed to form crRNA containing sequences matching exogenous genes. The crRNA recognizes the ho-

mologous sequence of the virus genome and mediates the binding and

* Corresponding author at: Biomedical Engineering Research Center, Kunming Medical University, Kunming, China.

** Corresponding author at: Guangdong Key Laboratory for Research and Development of Natural Drugs, The Marine Biomedical Research Institute, Guangdong Medical University, Zhanjiang, China.

E-mail addresses: zhangzhibi@kmmu.edu.cn (Z. Zhang), xzhu@gdmu.edu.cn (X. Zhu).

¹ Mingtao Zhang and Emily A. Eshraghian contributed equally to this work.

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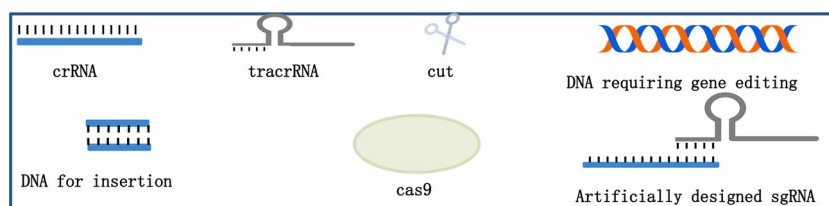
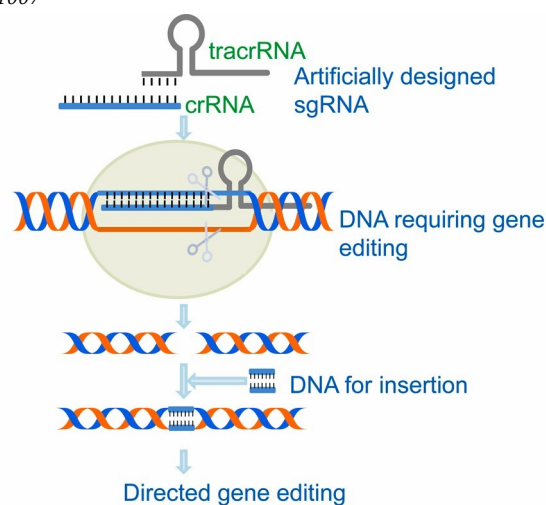


Fig. 1. The type II CRISPR/Cas9 system schematic diagram.

cleavage of the Cas protein (Fig. 2) [11–13].

Gene editing can be divided into three generations. Compared with the first and second generations of gene editing technology, CRISPR/Cas has its own unique characteristics [4,10,14–52]. Details are provided in Table 1.

The emergence of CRISPR/Cas gene editing technology provides new possibilities for human gene editing. With rapid development in recent years, it has been widely applied in many fields, especially in the fields of genetic disease treatment, disease-related gene screening and detection, tumor treatment, transformation of plants and animals, and prevention and control of pathogenic microorganisms, which have great potential and can effectively improve the quality of life of humans. However, CRISPR/Cas gene-editing technology is currently facing a series of difficulties, including but not limited to limited funding, ethical concerns, and cancer risk. To better understand the potential risks and benefits of CRISPR/Cas gene-editing, researchers will need to study this technology rigorously (Table 2) [53–63].

CRISPR technology can be used for both loss-of-function (such as CRISPR KO, CRISPRi) and gaining-of-function (such as CRISPRa) screening research, with powerful functions. The most common option is the CRISPR-KO knockout library, in which the Cas9 protein introduces double-stranded breaks in DNA, introducing code-shifting mutations through DNA repair based on non-homologous terminal connections (NHEJ), thereby disrupting gene function. However, gene knockout is not applicable in all scenarios. The regulation of gene expression and related phenotypic changes are the driving factors of many diseases. Thus, it may be more appropriate to up-regulate or down-regulate genes rather than completely knock them out, especially when studying non-coding and essential genes or looking at phenotypic changes at different levels of expression. The CRISPRa library includes CRISPR-dCas9-VP64, which functions to activate gene expression. The CRISPRi library includes CRISPR-dCas9-KRAB, which functions to silence or interfere with gene transcription. CRISPRa is

highly effective in the screening of functional gains. CRISPRi is highly effective in screening for missing functions; to note, it is more effective than the RNAi library that is currently well-utilized.

The use of CRISPR library: CRISPR libraries are used for gene screening. There are two main methods [64–67]. One is positive selection, which identifies the population with growth advantage through random mutagenesis. The other is negative selection, which identifies the genes necessary for survival under certain conditions. If a cell carries gRNA, which targets the genes necessary for survival, the knocked-out cell cannot survive.

There are two CRISPR/Cas9 library systems: the single-plasmid system and the dual-plasmid system. The single-plasmid system includes the promoter, gRNA, Cas9 and screening markers. Its advantage is that only one transfection is required and both gRNA and Cas9 are transferred. However, this system is too large, resulting in low infection efficiency. The dual-plasmid system is more efficient and easier to modify, as the plasmid contains only gRNA and the optimal promoter; this system needs to be transfected twice [68–70].

CRISPR has many advantages over other gene-editing techniques. In recent years, it has developed rapidly and been widely used in many fields [71]. This review mainly describes CRISPR gene editing technology and its research progress in the field of cancer.

2. New progresses

2.1. New generation CRISPR gene editing system: class 2 CRISPR-Cas systems

In 2015, Zhang et al. discovered a novel CRISPR system, the Cpf1 (Cas12a) enzyme system from *Acidaminococcus* and *Lachnospiraceae*. This system allows simpler and more accurate genomic engineering operations. More recent discoveries of Cas9 applications indicate that there are several

differences in Cas9 than what has been described (Table 3). Both Cas9 and Cas12a (Cpf1) have been successfully applied to genome engineering [72–76].

with RNA interference technology, cas13d-mediated gene silencing has higher specificity and knockout efficiency. Compared with cas9-mediated gene knockout technology, cas13d-

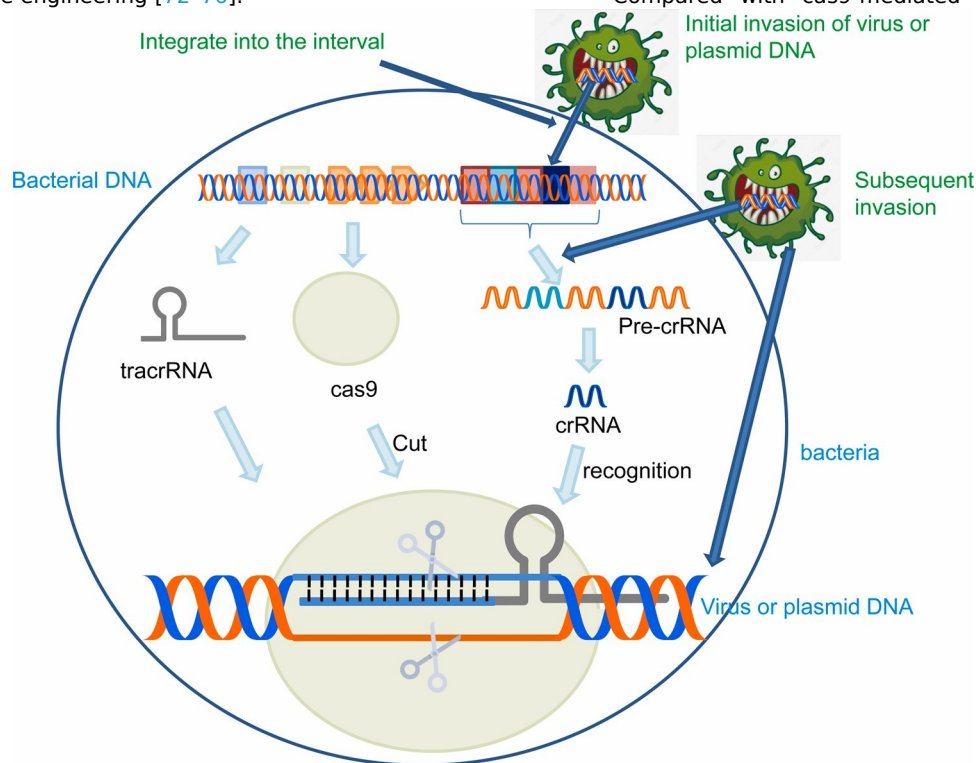


Fig. 2. The molecular mechanisms of II type CRISPR/Cas9 system diagram.

2.2. Research on the co-evolution of CRISPR-phage

When scientists use the CRISPR gene-editing tool to modify organisms or phages that affect them, they should consider the subtle interactions (dynamic evolution) between phages and bacteria. Data shows that combinations of encounter rates and mutation rates result in a five-state diagram of the complex interaction between CRISPR's addition-rejection rate and the encounter rate of bacterial and phages. As the encounter rate of bacterial and phages decreases, several states are found (Table 4). This indicates that a DNA CRISPR copy of a single virus is made only by reducing the rate of encounters between the phage and the bacterium, which in turn allows the bacteria to develop immunity against the phage that would eventually lead to its extinction. Though this is a landmark finding [77], it applies only to the use of CRISPR in bacterial and prokaryotic systems.

2.3. RNA targeting CRISPR system

Zhang et al. discovered two novel RNA targeting CRISPR systems. These new systems make use of Cas enzymes with similar properties, such as Cas13a and Cas13b. Cas13a, previously known as C2c2, is an RNA-targeting enzyme [78]. Cas13b has the ability to target and edit RNA; it only needs a single guide RNA to find the target, and it can also target multiple RNA transcripts. At the same time, it can code genetically. Cas13b also has some unique features that make it more suitable for fine-tuning gene function. This system will allow researchers to specifically manipulate RNA and use CRISPR in high-throughput ways to study a wide range of biological processes [79]. Currently, the Cas13d family, which was discovered in 2018, has been shown to interfere with RNA in mammalian cell lines to achieve gene silencing. Compared

mediated gene silencing does not alter genomic DNA, so this gene silencing is reversible and has significant advantages for the treatment of some acquired metabolic diseases. Compared with other Cas13 family proteins, CasRx has the highest RNA cutting activity and the smallest volume, making it easier to package into AAV for in vivo delivery. It is considered to be the most advantageous Cas13 protein in future applications [80,81].

2.4. New nanoparticle transport system

Researchers have developed novel nanoparticle transport systems, in which special vectors help CRISPR/Cas9 systems cross cell membranes into the nucleus and avoid being captured by organelles. CRISPR consists of two components, a scissor-like protein, known as Cas9, and an RNA molecule, known as sgRNA. The sgRNA molecule directs Cas9 to the target gene in the nucleus. Cas9 queries and fixes genetic errors in the nucleus to help host cells repair cellular machinery. This technique can overcome the transmembrane barrier of CRISPR gene editing and effectively improve its efficiency [79].

2.5. CRISPR-related transposase system - INTEGRATE

Screening gene editing elements allowed investigators to discover the Tn7 transposing factor of *Vibrio cholera*. In combination with the CRISPR system, Tn7 forms the CRISPR-related transposase system, referred to as INTEGRATE. The INTEGRATE system randomly integrates the inserted gene into various sites in the bacterial genome in positive and negative directions [82].

2.6. Diagnostic techniques

Four Cas systems are commonly used in CRISPR molecular diagnostic technology: Cas9, Cas12, Cas13 and Cas14 ([Fig. 3](#)). Several prominent scientists have made fundamental contributions to the invention and application of CRISPR technology and are actively committed to the industrialization of CRISPR technology [[10,78,79,83–106](#)]. Several diagnostic techniques are compared in [Tables 5 and 6](#).

Cas12a is the corresponding protein of Cas13a in DNA molecular

Table 1

Comparison of gene editing techniques.

Comparison of gene editing techniques.				
Gene editing technology	First generation	Second generation	Third generation	
Name	Homologous Recombination	Zinc finger ribonuclease (ZFN) [14]	TALEN [15]	CRISPR/Cas9 [10]
Year	1980s	1996	2011	2013
Structure		Consists of zinc finger DNA binding domain, which is responsible for specific recognition sequence, and DNA cutting domain, which is not specific restriction enzyme cutting [49].	Consists of an N-terminal domain containing a nuclear localized signal, a central domain containing a typical tandem TALE repeat sequence that recognizes a specific DNA sequence, and a C-terminal domain with the function of FokI endonuclease [48].	Composed of CRISPR sequence elements and Cas gene family [52].
Mechanism of action	Knock-out (i.e. removal of certain genes from original genome). Knock- in (i.e. integration of a gene at a site in genome that does not already exist). Knock-out and knock-in are performed by DNA homologous recombination.	ZFN is a synthetic restriction enzyme that plays a role through its zinc finger DNA binding domain and DNA cutting domain.DNA binding domain is modified to target different DNA sequences, and then DNA cutting domain was used for specific cutting. ZFN technology can also work with the intracellular DNA repair mechanism, enabling easy genome editing [50].	Can specifically bind to the target sequence. By linking an artificial TALE to a FokI nuclease, it forms TALEN, a powerful genome-specific editing tool [48].	Cas9 crRNA and tracrRNA form a complex. The Cas9 protein cuts the double-stranded DNA at the target site of the crRNA-paired sequence. Through artificial design, crRNA and tracrRNA can be modified to guide sgRNA, guide Cas9 to cut DNA at a fixed point, and finally achieve gene knockout and insertion [9,10,52].
Endonuclease		Fok1	Fok1	Cas
DNA-specific recognition region		Zinc finger protein ZF	TALE factor	crRNA or sgRNA
Identify target size		(9 or 12bp) *2	(8– 31bp)*2	20bp + NGG
Difficulty and speed of design process	Difficult & timely	Difficult & timely	Most difficult & timeliest	Easy & quick
Cost		High	Lowest	Low
Off-target effects		Lowest	Low	High
Efficiency		High	High	Highest
Methylation sensitivity		Sensitive	Sensitive	Not sensitive
Application	Establishing animal models for the study of genetic diseases. Knockout. Knock-in.	Used to knock out and deactivate genes, either to introduce target genes to activate or block them, or to engineer gene sequences to meet human requirements. In the medical field, plasmids or stem cells modified by ZFN technology to introduce therapeutic genes can be introduced into human body to achieve gene therapy, and can also be directly used to repair, replace or delete harmful genes to achieve relevant therapeutic purposes [51].	Used to target genes. Increasingly used in combination with microinjection, lentiviral infection and other techniques.	Used in genetic modification, animal model construction, drug research and development, gene therapy, agricultural breeding, etc.
Advantages		Platform maturity. High efficiency. Can be designed for specific sequences to modify target genes. High specificity, avoiding the immune response.	Design and construction screening is simpler than ZNF. High specificity. Off-target effects are extremely low. Lower cost than ZNF. Easier to use than ZNF.	Simple design. Low requirements on target sequence, accurate targeting, good adaptability and more target genes. Simple, cost-effective and efficient to construct short RNA sequences complementary to the target sequence. Suitable for multigene, high-throughput operation and has a wide range of applications.
Limitations	Difficult to use in clinical settings or widely in agriculture and animal husbandry. Low probability of genetic recombination. Steps are complicated, time-consuming, and costly.	Inefficient. Specificity of DNA sequence recognition is poor. Genetically engineered zinc finger DNA binding protein is difficult. Costly.	Assembly of LEN - encoded plasmids is lengthy and high - strength. Diversity is difficult.	Compared with ZNF and TAFEN, specificity is low. Off-target effects are high. NHEJ still produces random toxicity.

recognition, shearing and degradation. Researchers introduced RPA on the basis of Cas12a as a new nucleic acid molecular detection technology, named DETECTR. Compared with SHERLOCK, DETECTR eliminates the step of transcription of amplified DNA products into RNA [86].

Based on the Cas13a system, Zhang et al. introduced RPA, the technology of normal temperature amplification of DNA, and created a new technical platform, known as Specific High Sensitivity Enzymatic Reporter UNLOCKing (SHERLOCK). The core principle of SHERLOCK technology includes site-specific amplification: if DNA is tested, it is amplified by RPA; if RNA is tested, it is amplified by RT-RPA. The amplified DNA products are then transcribed into RNA molecules. Target RNA was detected by Cas13a-crRNA and reporter RNA [86,94]. Due to its high sensitivity and specificity, this CRISPR diagnostic technology has many uses.

Researchers found that Cas14 in the gene bank has four characteristics. First, its small size makes it relatively easy to produce on a large scale. Second, only single-stranded DNA (ssDNA) is targeted; after activation, other ssDNA is cut nonspecifically in addition to the target ssDNA. According to this feature, Cas14-DETECTR was designed. Third, the recognition sequence has no PAM restriction and can target any sequence. Fourth, Cas14-DETECTR achieved DNA detection with single- base resolution. Due to this feature, high-fidelity SNP genotype analysis method was established with Cas14-DETECTR [93].

Researchers developed HUDSON technology for real-time virus detection. Through heating, the following steps occurred in order: the nuclease was inactivated, the virus was inactivated, and there was diagnosis by SHERLOCK.

Table 2
The emergence, development and current situation of CRISPR/Cas9 system.

Source(s)	Content
emergence	
[56]	The CRISPR sequence, a particular repetitive interval sequence, has been identified for the first time in the genome of <i>Escherichia coli</i> .
[57,58,59]	Francisco Mojica, who first named CRISPR, found that there were interrupted repeats in more than 20 microbes.
[60]	The molecular mechanism of the CRISPR/Cas system was revealed.
[4]	The CRISPR/Cas9 system was found to be efficient at editing genomes.
development	Use of CRISPR-Cas9 to treat Sickle cell disease (treated with CTX001) and cystic fibrosis.
2015– 10	CRISPR Therapeutics announced a partnership with Vertex Pharmaceuticals.
2015-12-21	CRISPR Therapeutics announced a joint venture with Bayer called Casebia Therapeutics.
2016-6-8	CRISPR Therapeutics has announced a partnership with Anagenesis Biotechnologies.
[78]	To obtain a license to edit paraxial mesoderm multipotent stem cells. Based on the technology, CRISPR therapeutics could effectively differentiate pluripotent stem cells into skeletal muscle stem cells, leading to the efficient development of CRISPR therapies for Duchenne muscular dystrophy.
[83]	CRISPR/Cas13a was found to have specific RNA sequences that cut through bacteria.
[98]	Confirmation that CRISPR/Cas13a can be used for RNA testing.
	CRISPR/Cas9 gene editing applied to CAR-T therapy using adeno-associated virus-mediated gene editing. This study not only solves the potential harm that may exist in the random integration of traditional CART therapies, but also greatly reduces the risk of differentiation or carcinogenesis of CAR-T cells, giving the CAR-T technology a new efficiency, stability and safety.
[99]	The use of CRISPR/Cas9 allowed modification of a mutation linked to genetic heart disease in human embryos before implantation in the womb. The study confirms the safety and feasibility of editing human embryos to treat genetic diseases. However, the work has been questioned by others in the field of gene editing.
[100]	The endogenous retrovirus (PERV) sequence in the pig genome was knocked out by CRISPR/Cas9 technology and multiple PERV inactivated piglets were cloned. A big step towards the ultimate goal of using pig organs for human organ transplantation.
[63]	Academician Longping Yuan, father of hybrid rice, announced the success of rice breeding using CRISPR/Cas9 technology to knock out genes related to arsenic uptake and accumulation. This study fundamentally solves the problem of arsenic pollution in rice, will reverse the problem of excessive heavy metals in some crops in China, and further improve the problem of chronic heavy metal poisoning in humans.
[101]	It was demonstrated that CRISPR/Cas13a could edit specific RNAs in mammalian cells. CRISPR/Cas13a could achieve similar efficiency to RNAi in reducing gene expression, with greater specificity and less impact on the natural post-transcriptional regulatory network in cells.
[102]	A highly accurate variant of Cas9, HypaCas9, was designed. This study significantly reduced the off-target effect of Cas9 without reducing the efficiency of targeted cutting.
[103]	This paper introduces a new CRISPR system, REPAIR, which can efficiently repair single-base RNA. By not altering the DNA sequence, it opens up new possibilities for gene editing to treat genetic diseases without permanently affecting the genome.
[104]	Reported the new editor - ecTadA - dCas9 adenine gene (A). T base pairs can be converted into A, G, C base pairs. The technology for the first time can be achieved without relying on DNA gene editing technology, namely the disease- related gene editing techniques. The technology is higher than other genome editing method is inefficient and almost no adverse side effects such as random insert, delete, or other mutations, so A wide range of treatment for the future point mutation genetic diseases provides A great convenience.
2018-9-17	ViaCyte pioneered ways to generate pancreatic cells from stem cells and use them safely and effectively in patients. CRISPR Therapeutics' CRISPR gene-editing expertise has the potential to protect transplanted cells from type 1 diabetes patients' immune systems through in-vitro editing. They developed gene editing stem cell derived therapies for diabetes.
2019-1-4	The FDA has added CTX001 to its fast-track approval pipeline, which will speed up the drug approval process and bring it to market. Editas is developing drugs for duchy's muscular dystrophy, cystic fibrosis and cancer, but the difference is that it initially focused on treating eye diseases with CRISPR, such as the inherited primary cause of childhood blindness known as LCA10.
current situation	
[55]	Reports results of gene modification in human embryos using CRISPR gene-editing technology.
[61][62].	Demonstrates ability to use CRISPR to knock out HIV from human T-cells in vitro.
[143]	This paper was quickly retracted after determination of no conclusive evidence that the mutation was caused by CRISPR.
[8]	The first pair of gene-edited children born, claiming natural immunity to HIV.

The researchers combined CRISPR and graphene membrane field effect transistor technology to create the CRISPR-Chip technology. The graphene-based transistor contains a complex of dCas9 (recognition only, no shear) and sgRNA. When a sample is dribbled onto a graphene membrane, dCas9-sgRNA recognizes and binds to the corresponding target DNA molecule, and the transistor sends out an electrical signal. In SHERLOCK and DETECTR, "cut" generates signals; in CRISPR-Chip, binding generates signals. Compared with SHERLOCK and DETECTR, the CRISPR-Chip technology is advantageous in that the DNA to be tested does not need amplification; however, special instruments are needed to facilitate this technology [87].

Table 3
Differences between Cpf1 (Cas12a) enzyme system and Cas9 enzyme system [73,75].

Advantages of Cpf1 enzyme system	Results
The Cpf1 system is simpler, requiring only one RNA, and smaller than the standard SpCas9.	The Cpf1 system is easier to deliver to cells and tissues.
Cas9 complex cuts the same site of two DNA strands, leaving blunt ends that will mutate when reconnecting, while Cpf1 complex cuts the two DNA strands with offset incision, leaving overhang at the exposed end, which is not easy to mutate.	The Cpf1 system is able to integrate a piece of DNA more efficiently and accurately.
The Cpf1 incision is far away from the recognition site, and if there is a mutation in the target gene at the cutting site, it can still be recut.	The Cpf1 system provides the opportunity to correct editing.
Like Cas9, the Cpf1 complex must first attach a short sequence of PAM, but the Cpf1 system recognizes a distinct sequence of PAM from Cas9.	Cpf1 system has the flexibility to select target sites.

Table 4
A five-state diagram of the co-evolution of CRISPR-phages [77].

State	Meet rate	Bacterial DNA mutation rate	The relationship between bacteria and phages
Fifth state	+++++	+++++	Phages are more likely to escape
Fourth state	++++	++++	Phage will escape without becoming extinct
Third state	+++	+++	Bacteria coexist with phages
Second state	++	++	Phages fall into extinction
First state	+	+	Phage extinction is even greater

Note: The '+' notations indicates probability (i.e. more '+' notations are equal to higher probability of event).

2.7. Add a switch to Cas9 to make genome editing safer

Cas9 is always turned on, leading to off-target effects that make it difficult to achieve cell or tissue specificity. The researchers used circular permutation to modify Cas9 to produce four Cas9-CP variants, namely CP199, CP1029, CP1249 and CP1282, that bind to and cut DNA in a way that is equivalent to the wildtype version. Cas9-CP199, which is called Cas9-CP (ProCas9), senses and responds to protease activity. The mechanism of ProCas9 is as follows: the joint sequences are spatially inhibited so that they exist in the cell in an inactive state. The homologous protease that identifies the joint sequence can alleviate inhibition by targeted cutting, thus restoring the activity of ProCas9. This will make CRISPR-Cas genome editing safer [107].

2.8. Cryogenic electron microscope structure of the Csm-crRNA complex

Certain bacteria use type III CRISPR-Cas in Csm (III - type A) or Cmr (III - type B) to synergize the effect complex cracking invasion of ssRNA and ssDNA substrates, including ssRNA combined with crRNA as its complement. The coupling of ssRNA cleavage with DNase activity in III- A CRISPR-Csm was demonstrated by cryo-EM and biochemical methods [108].

2.9. Structural mechanism of xCas9 broadened PAM compatibility and high DNA fidelity

In order to overcome the off-target effect of the enzyme SpCas9 in the CRISPR-Cas9 system, the researchers proposed a crystal structure of the SpCas variant xCas9 3.7, and revealed the molecular mechanism of the compatibility and high fidelity of the substrates identified by the SpCas9 variant (xCas9 3.7) through structural biology and biochemical research [109].

2.10. A third universal CRISPR tool: CasX

The researchers demonstrated that CasX is an RNA-guided DNA endonuclease, also known as Cas12e5. Due to its small

size (<1,000 amino acids), DNA cutting characteristics, and its derivation from non- pathogenic microorganisms, the enzyme family is distinguished from Cas9 and Cas12a. CasX has evolved into a third powerful tool for RNA programming genome editing [110].

3. Cancer treatment applications

3.1. Using CRISPR to transform cancer cells

CRISPR may be utilized to remove cancer cells that have left the original tumor and metastasized to other parts of the body. They were engineered using the genome-editing technique CRISPR. Cancer cells are first harvested from individuals with the disease and grown in culture. They then used CRISPR gene-editing technology to knock out the cancer cell's death receptor gene. The ligand molecules that induce death and the genes that turn GCV (Ganciclovir) into a cancer-killing drug were then transferred to these cells. The modified cancer cells are then injected into the circulatory system. The CRISPR-engineered cancer cells, based on their homing properties, were able to attack and track both in situ and metastasized tumors and eventually self-destruct, which may have plausible impact for the survival of cancer patients [111].

3.2. Use CRISPR to construct cancer-like organs

Colorectal cancer usually develops slowly from an adenoma and is likely to be due to the acquisition of genetic mutations. Using the CRISPR/Cas9 technique in vitro for cultured human intestinal stem cells, researchers introduced four of the most common colorectal cancer gene mutations (APC, P53, KRAS, and SMAD4). The mutated cells were then screened by removing growth factors from the medium to construct CRC like organs. By modeling cancerous organs through the induction of mutational signatures, we are able to utilize CRISPR to more adequately investigate cancer. Other cancer-like organs could also be built for research [112].

3.3. Reveal the mystery of cancer immunotherapy

Immunocheckpoint inhibitors are novel mechanisms used in immuno-oncology. Some patients respond to immunocheckpoint suppression therapy, while others do not [113]. Using genome-wide CRISPR screening, the researchers

found that patients whose tumors expressed genes encoding specific proteins were able to respond to cancer immunotherapy, indicating that some oncogenes are essential components of immuncheckpoint inhibitor therapies. Researchers used CRISPR-Cas9 to develop a new screening method that could be used to detect the function of thousands of tumor genes in mice. Using this screening method, the team identified a new drug target, Ptpn2, which is expected to enhance the effectiveness of PD-1 checkpoint inhibitors, a key member of cancer immunotherapy [114,115]. A series of in-depth experiments demonstrated that gamma interferon enhances the ability of T-cells to find tumor cell targets and

APLNR, a gene encoding the G-protein-coupled apelin receptor, regulates the cytokine gamma interferon signaling in tumor cells, which increases the activity of genes involved in antibody processing and presenting [116]. Several other genes are involved in this mechanism. For example, SOX10, a gene that encodes transcription factors involved in embryonic development, is overexpressed in melanoma. The gene for Bardet-Biedl syndrome 1 may play an important role in the development of several organs, including the heart. RPL23 and RPL10 which encode the 60S ribosomal subunit

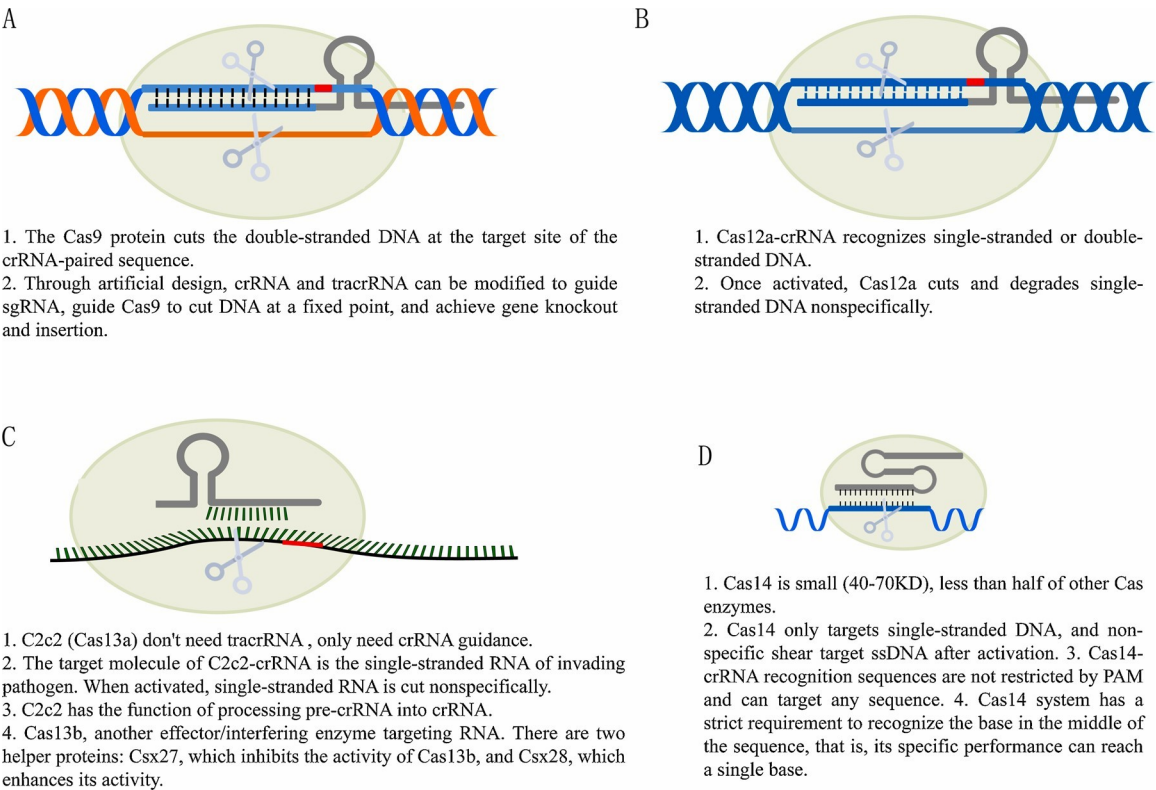


Fig. 3. Gene-editing schematic diagram of four Cas systems (Cas9, Cas12, Cas13, Cas14) commonly used in CRISPR molecular diagnostic technology.

A: Gene-editing legend of the CRISPR/Cas9 system.
B: Gene-editing legend of the CRISPR/Cas12 system, similar in principle to the CRISPR/Cas9 system.
C: Gene-editing legend of the CRISPR/Cas13 system, similar in principle to the CRISPR/Cas9 system.
D: Gene-editing legend of the CRISPR/Cas14 system, similar in principle to the CRISPR/Cas9 system.

Table 5
Four Cas systems commonly used in CRISPR molecular diagnostic technology (Cas9, Cas12, Cas13, Cas14).

The Cas system	Cas9	Cas12	Cas13	Cas14
Graphic	Fig. 3	Fig. 3	Fig. 3	Fig. 3
Discovery	In 1987, the CRISPR sequence was first identified in the genome of <i>E. coli</i> . In 2001, CRISPR was first named by Francisco Mojica, who discovered the presence of interrupted repeats in more than 20 microbes. In 2011, the molecular mechanism of the CRISPR/Cas system was revealed. In 2013, the CRISPR/Cas9 system was found to be efficient at editing genomes.	Doudna et al. discovered that Cas12 is responsible for Cas13a's molecular recognition, splicing, and degradation of DNA [94].	Zhang's team searched the gene bank for Cas1 gene and then found genes associated with Cas1. After several rounds of screening and verification, they found three new second-class systems: C2c1, C2c2 (later named Cas13a), and C2c3. Using a similar approach, they found Cas13b, another effector enzyme that targets RNA [79, 87].	The Doudna team searched the gene bank for the Cas1 gene and found the gene associated with Cas1 on the DETECTR technology platform [91].
Target	Double-stranded DNA	Single-stranded or double-stranded DNA	Single-stranded RNA	Single-stranded DNA
Requirements for PAM	High	Medium	Low	No
Cut Size	Selectively Large	Indiscriminately Large	Indiscriminately Large	Indiscriminately Small
tracrRNA	Need	Need	Unneeded [78]	Need
Diagnostic techniques involve:	CRISPR-Chip [85] CRISDA [97]	DETECTR [94,95] HOLMES [96] SHERLOCKv2	SHERLOCK [84,92] [95], SHERLOCKv2	Cas14-DETECTR [91]

protein components were also implicated [116]. Through these studies, we have a better understanding of how cancer cells respond to T-cells and why they become resistant, with

the ultimate goal of using the genes involved in cancer immunotherapy and turning non-responsive patients into responsive ones.

Table 6

CRISPR molecular diagnostic technology.

Diagnostic techniques	Source(s)	Application	Principle	Strengths	Limitations
DETECTR	Cas12a + RPA [94,95]	Nucleic acid molecular test	When Cas12a detects the target DNA sequence, it will cut the fluorescence report sequence and release the fluorescence signal to achieve the detection purpose.	Omits transcription from amplified DNA products to RNA. DETECTR achieved aM level sensitivity and specificity of ≤ 7 bases. Fast, accurate, easy to use, and cost-efficient. Suitable for mass screening of HPV.	
SHERLOCK	Cas13a + RPA [84,92,95]	Used to detect specific nucleic acid molecules, virus detection and virus subtype differentiation, pathogen identification, drug resistance gene identification, instant detection (POCT), patient genotyping, cancer mutation analysis and liquid biopsy.	The nucleic acid molecule to be tested is first amplified; DNA amplified by RPA and RNA amplified by RT-RPA. The amplified DNA products are then transcribed into RNA molecules. The detection of target RNA is accomplished by Cas13a-crRNA and report RNA.	High sensitivity and specificity allows single- base resolution, depending on crRNA design. All reactions can be done in one solution and all reagents can be turned into freeze- dried powder and fixed on the reagent paper, which does not affect the sensitivity of detection.	Potentially inconvenient, as virus testing requires the purification of viral RNA from patient's body fluid sample.
SHERLOCK in gold standard chromatography format	Improved SHERLOCK gains [84]	It can be used for quick liquid biopsies in cancer patients. Viral RNA can be detected at concentrations as low as 2 aM.	Luciferin and quencher at both ends of the RNA reporter were replaced with biotin and FAM, respectively. If the RNA reporter is not cut, the gold nanoparticle -FAM antibody - FAM-RNA reporter-biotin coupling is stranded on the first line by streptavidin, and the second line does not show color. If the RNA reporter is clipped and the gold nanoparticle -FAM antibody-FAM is released, it can run to the second line and be trapped by protein A. The positive or negative result of the diagnosis can be read by the second line of chromogenic or non-chromogenic interpretation.	There are no restrictions on its use based on various settings.	
HUDSON	[86]	Real-time virus detection	The nuclease is inactivated by heating (plus TCEP/EDTA), then the virus is inactivated, and finally diagnosed by SHERLOCK.	All reactions take place in a single container and do not require fluid exchange or purification. The step of nucleic acid extraction in routine testing is omitted.	
Cas14-DETECTR	Cas14+ DETECTR [91]	Rapid and efficient diagnosis of infectious diseases, cancer and other diseases.	Cas14-sgRNA recognizes only single-stranded DNA, and RPA amplification products need to be converted to ssDNA. At DNA amplification, only one of the primers contained phosphorothioate (PT). After amplification, DNA strands not protected by PT were degraded by T7 exonuclease, while the PT-protected single strands survived and continued to be screened by cas14-sgRNA.	Cas14a is good at identifying ssDNA, so RNA, dsDNA and ssDNA can all be tested. The increase of specificity can detect high fidelity SNP genotyping.	
CRISPR-Chip	CRISPR + gFET [85]	It can be used to quickly diagnose diseases or evaluate the accuracy of	The graphene-based transistors carry a complex of dCas9 (recognition only,	Fast, efficient, no amplification required.	Compared to SHERLOCK and DETECTR, the

	gene-editing techniques.	no shear) and sgRNA. When the sample is dripped onto the membrane, the transistor emits an electrical signal once dcas9-sgRNA recognizes and binds to the corresponding target DNA molecule.	CRISPR-Chip technology requires special instruments.
HOLMES	Cas12a + PCR [96]	It can be applied to the detection of pathogens, cancer mutations, single nucleotide polymorphisms, genetically	Fast, sensitive, high - specific, simple, low - cost, without professional equipment.

(continued on next page)

Table 6 (continued)

Diagnostic techniques	Source(s)	Application	Principle	Strengths	Limitations
CRISDA	CRISDA was activated by the use of a unique conformational change in the CRISPR systemic effect protein Cas9 during binding to a target nucleic acid molecule [97].	modified food and other fields. The exponential amplification of target nucleic acid molecules can be achieved efficiently.		Time-efficient, without any temperature change process. The sensitivity, specificity, universality and operability are better than that of traditional PCR.	

3.4. CRISPR combined with CAR-T to conquer cancer

3.4.1. Construction of more potent chimeric antigen receptor T cells (CAR T-cells)

CAR T-cell therapy is a form of therapy that utilizes CRISPR/Cas9 technology for delivery of CAR genes to specific sites in the T-cell genome. This allows construction of CAR T-cells that are unable to be depleted and can kill tumor cells for longer periods of time. It provides the possibility for effective treatment of cancer in humans [100,117, 118].

3.4.2. CRISPR is combined with universal CAR-T

Most CAR T-cells are obtained from patients' own T-cells, so the production of universal CAR-T is of great significance for cancer patients. The researchers used CRISPR to knockout two genes involved in immune exclusion: T-cell receptor and Beta-2-Microglobulin. This knockout resulted in reduced alloreactivity and absence of graft-versus- host disease. This mechanism serves to reduce the antigenicity of CAR-T and obtain universal T-cells. In addition, universal CAR-T is thought to be the same type of T-cell that can load different antigens. Based on this, the researchers developed a separate, universal, programmable CAR system for T-cell therapy called SUPER CAR-T. That is, patients with different cancers only need to input the same SUPER CAR-T, which can simplify and accelerate treatment [119-124].

3.4.3. Combination of CRISPR and enhanced CAR-T

Using CRISPR to knock out genes in immune co-inhibitory pathways, or signaling molecules, ultimately improves the efficacy of CAR-T cells [125,126]. In short, CRISPR combined with CAR-T to combat cancer demonstrates a synergy.

3.5. Enhance the anti-cancer ability of T cells

Researchers used CRISPR genome-wide genetic screening to systematically study the molecular mechanism of T-cell activation and to map the regulatory atlas of human T-cell function. At the same time, it was found that FAM49B, a gene with unknown function, could affect cytoskeletal rearrangement of T-cells by activating Rac in T-cells. If knocked out, T-cells would be over-activated and actin aggregation would be enhanced, such that T-cell activity

would be upregulated. This suggests that FAM49B may be a novel target of tumor immune drugs that can enhance the anti-cancer ability of T-cells [127].

3.6. Looking for cancer's "mutation label"

The accumulation of mutations in genetic material usually results from the following causes: initial environmental problems, changing of enzyme activity, failure in DNA replication or DNA repair mechanisms, and more. The initial mutagenesis condition that leads to the formation of their own DNA damage patterns is referred to as a mutational signature. Researchers have developed a new experimental device that can examine mutation labels in cell cultures. The study confirmed an analytical strategy for describing mutation processes and cancer development, and also demonstrates that mutation labeling is a direct output mechanism of cell-specific dysfunction. Therefore, mutation labels can be used as biomarkers for tumor molecular characterization, and the use of this new diagnostic tool can improve the accuracy and personalized treatment of cancer [128,129].

3.7. Use CRISPR/Cas9 to mutate or inactivate cancer cells genes

The CRISPR/Cas9 system can cut and modify targeted mutations, as well as specifically target a range of common cancer mutations in healthy wild-type alleles that are not clearly targeted. The expression of Cas9 enzymes that carry cancer-specific guidance for RNAs can also uncover mutations that lead to cancer cell growth and gene mutations. Eventually, genes in cancer cells can mutate or become inactivated, indicating effective treatment [130].

3.8. Genome-wide screening using CRISPR-Cas9 to find tumor weaknesses

The researchers conducted genome-wide CRISPR-Cas9 screening in RNF43 mutated pancreatic ductal adenocarcinoma (PDAC) cells. Through screening, they discovered that a Wnt signaling pathway involving FZD5 plays an important role in the proliferation of PDAC cells with RNF mutation. FZD5 is one of the 10 Frizzled receptors encoded in

the human genome. Identification of the weakness of pancreatic tumors leads to potential antibody drugs that could be used to treat this type of cancer [131]. Acute myeloid leukemia (AML) is an invasive blood cancer marked by AML cell proliferation, which interferes with the bone marrow's ability to make normal blood cells. The CRISPR-Cas9 technology was used to destroy all the genes in the AML cell genome and to identify genes that are harmful to the growth and survival of AML cells after the destruction [132]. Large-scale screening with CRISPR/Cas9 technology revealed that histone demethylase, a tumor suppressor gene, promotes the development of lung cancer when inactivated, thus providing a potential therapeutic strategy [133]. CRISPR-Cas9 can be utilized to conduct genome-wide screening for tumor weaknesses, which can be exploited to attack the tumor to provide effective treatment.

3.9. Use CRISPR-Cas9 to select the first priority target for cancer treatment

This study conducted genome-wide CRISPR-Cas9 cell function screening for 324 tumor cell lines and integrated analysis to screen out priority targets for cancer treatment, which has important practical significance for the development of diversified and more effective new anticancer drugs [134].

3.10. Screening of cancer drug targets by CRISPR system

CRISPR-Cas9 technology was applied to target the coding exon of the protein's functional domain, so as to conduct large-scale screening of cancer drug target. It is of great significance for cancer drug target screening [135].

3.11. New targeted drug delivery system for tumors

Liang et al. developed a tumor-targeted delivery of CRISPR/Cas9 utilizing aptamer-functionalized lipopolymer to achieve selective genome editing in tumors. They demonstrated selective distribution of CRISPR/Cas9 in osteosarcoma in situ and pulmonary metastasis in a mouse model, effectively editing vascular endothelial growth factor A, VEGFA, and thus inhibiting malignant OS in situ and lung metastasis. Therefore, conjugation to natural products of plant origin with high cytotoxic activity can allow for specific identification of target cancer cells; this paves the way for a new generation of smart tumor targeting compounds, namely aptamer-drug coupling. This provides a solution to the CRISPR/Cas9 in-vivo targeted delivery problem [136,137].

3.12. Use CRISPR to screen new cancer targets for cancer treatment

It was found that Np63a, an oncogenic product expressing the p63 gene, was ubiquitous in squamous cell carcinoma. Np63a suppresses the expression of two tumor suppressor genes, TGFB2 and RHOA, further promoting cancer progression. Therefore, finding a way to reactivate the TGFB/RHOA pathway could save the lives of cancer patients [138].

3.13. KRAS drives new cancer drug targets

KRAS gene mutation-driven cancers are the most fatal type, with about 20–30 % of all human cancers being related to KRAS gene mutations. These include lung, pancreatic and rectal cancers, which are the most difficult to treat [139–141]. The team used CRISPR-Cas9 gene editing to systematically inactivate every gene in the human colon cancer cell genome. Inactivated NADK and KHK genes (which encode metabolic

enzymes) reduced KRAS mutated colon cancer cell growth in mice by about 50 %, but with potential limitations of standard laboratory cancer cell growth tests. Researchers used CRISPR-Cas9 to inactivate each gene in human colon cancer cells with normal KRAS and mutated KRAS, respectively, to find the combined efficacy of NADK and KHK, namely, the inactivated two genes, normal KRAS tumors were not affected, but the growth of mutated KRAS tumors decreased by nearly 50 %. Tumor suppressors have been found to inhibit tumor growth; one example is the INO80C encoding gene, functions to stabilize the genome and allows tumor suppression [142].

4. Safety issues

4.1. Cancer risk

The p53 gene plays a vital role in the projection of gene editing. This allows partial explanation for why CRISPR/Cas9-mediated partial cell editing is inefficient. In the presence and functioning of p53, CRISPR can activate p53 and cause DNA damage, resulting in failed gene editing or self-destruction of cells. Deletion or mutation of p53 or dysfunction of the p53-p21 pathway may result in efficient gene editing, but these cells are at risk of becoming cancerous [143–148].

4.2. Off-target effects

Several studies have revealed that Cas9-induced DNA double-strand breakage has a global destructive effect on the human genome, indicating that this breakage may result in serious consequences during clinical application. It is important that prior to any clinical application of CRISPR gene editing, there is assessment of the carcinogenic risk in order to prevent CRISPR-induced double strand breakage [149–152]. The goal of treatment is to cut off pathogenic genes without disrupting or affecting other genes (e.g. p53). Therefore, methods for sequencing and monitoring cells before and after editing, as well as methods to monitor the actual editing process, including the involvement of DNA breakage, still need to be explored and developed [153,154].

4.3. Ethical and moral issues of gene editing

Currently, the ethical issues surrounding gene editing fall into three main categories: enhancement features, medical benefits, and unmet medical needs [155–157]. Enhancement features are traits that cultivate ideals and are considered unethical in three potential applications, but are analyzed on a case-by-case basis. There exist ethical concerns regarding gene editing for the purpose of boosting a child's intelligence, yet common consensus indicates that it is ethically plausible to use gene editing technology to restore the health status of a child with muscular dystrophy. Second, though medical benefits have unlimited potential, the risks are unknown. It is difficult to make a moral case for germline editing. Third, unmet medical needs apply in very rare cases where the only option for building healthy children is via gene editing [8,158]. If the parents have a chromosomal recessive disease, the technology can be used in advance to allow birth of a child who is projected to have better health outcomes. In reality, however, there are concerns about whether germ-cell editing causes off-target mutations in cancer or other diseases; it is unknown whether these risks are passed on to the next generation. A robust debate on how to use germ cell editing in an ethical and safe setting is needed to pave a way forward.

5. The limitations and difficulties of CRISPR in cancer therapy

It is only in the last few years that CRISPR gene editing technology has been applied to gene modification in eukaryotic cells, and there are still some improvements in this technology.

One of the most promising applications of CRISPR/ Cas9 in gene therapy is CAR T cell therapy. However, clinical studies have found that this treatment is neurotoxic in vivo and may cause cytokine release syndrome. The CAR T cell clinical trial involves collecting T cells from patients, genetically modifying them and sending them back to them for therapeutic purposes. The technical requirements for medical equipment and personnel are extremely high. It is also difficult to get enough functioning autologous cells from patients. Editing allogeneic T cells from a single donor to produce "universal" CAR T cells and then administering them to multiple other patients has become a new line of research [159-163].

In terms of increasing the specificity of CRISPR gene modification, it is mostly through changing the activity and sensitivity of Cas9 nuclease, such as Cas9 notch (NICKase), which only performs single-stranded notch cutting of double-stranded DNA.

The use of CRISPR to prevent human diseases still requires the use of viral vectors as a means of delivering DNA. The AAV vector systems are commonly used in gene therapy may also have problems such as delivery efficiency and safety.

The modification of eukaryotic genes using CRISPR technology is based on the principle of homology - directed repair (HDR). The HDR gene is active only in dividing cells, and most human tissue cells are non-dividing cells. The search for mechanisms and methods to activate HDR- related genes in non-dividing cells will help the CRISPR technology to effectively modify HDR in such cells [164].

6. Perspectives

In recent years, CRISPR/Cas gene editing technology has made rapid developments and been widely applied in many fields, especially in medicine, where it has great potential. There are several potential benefits and limitations to CRISPR/Cas gene editing technology. Nevertheless, scientists will continue to investigate this technology in a rigorous manner to advance research and improve quality of life for humanity [155,158,165,166].

7. Conclusions

The emergence of CRISPR gene editing technology provides a reliable method for genome analysis, regulation, and editing.

It enables the application of biological research and biotechnology to be carried out in a wide range of fields.

Scientists have made a series of breakthrough research achievements based on CRISPR gene editing technology, which has the potential to drastically impact treatment and prognoses of patients with cancer. In recent years, this technology has attracted the attention of a large number of researchers, who have discovered many new developments in the process of in-depth study of this technology, making CRISPR gene editing simpler, more precise, and more detailed.

While literature indicates the potential benefits of this unprecedented technology, there may be some accompanying risks. Concerns surrounding cancer risk and ethical/moral issues are warranted and worthy of further consideration by researchers. Until then, future researchers will not be able to move forward as it is crucially important to first identify potential risks and find appropriate solutions [167,168].

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Declaration of Competing

Interest None.

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